

Mechanism of Inhibition of Deoxyribonucleic Acid Synthesis in *Escherichia coli* by Hydroxyurea

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The effects of hydroxyurea on *Escherichia coli* B/5 physiology (increases in cell mass, number of viable cells, and deoxyribonucleic acid [DNA], RNA, and protein concentrations) were studied in an attempt to find a concentration that completely inhibits DNA synthesis and increase in number of viable cells but has little or no effect on other metabolic processes. These conditions were the most closely approached at an hydroxyurea concentration of 0.026 to 0.033 M. A concentration of 0.026 or 0.033 M was used in subsequent experiments to study the site(s) of inhibition of DNA synthesis in *E. coli* B/5 by hydroxyurea. Hydroxyurea at a concentration of 10^{-2} M was found to inhibit ribonucleoside diphosphate reductase activity completely in crude extracts of *E. coli*. The synthesis of deoxyribonucleotides was greatly reduced when *E. coli* cells were grown in the presence of 0.033 M hydroxyurea. Studies on the acid-soluble DNA precursor pools showed that hydroxyurea causes a decrease in the concentration of deoxyribonucleoside diphosphates and deoxyribonucleoside triphosphates and an increase in the total concentration of ribonucleotides. Sucrose density gradient sedimentation of DNA from cells treated with 0.026 M hydroxyurea for 30 min indicated that at this concentration hydroxyurea induces no detectable single- or double-strand breaks. In addition, both replicative and repair syntheses of DNA were found to occur normally in toluene-treated cells in the presence of relatively high concentrations of hydroxyurea. Pulse-chase studies showed that deoxyribonucleotides synthesized prior to the addition of hydroxyurea to cells are utilized normally for DNA synthesis in the presence of hydroxyurea. On the basis of these observations, we have concluded that the primary, if not the only, site of inhibition of DNA synthesis in *E. coli* B/5 by low concentrations of hydroxyurea is the inhibition of the enzyme ribonucleoside diphosphate reductase.

Hydroxyurea (HU) has been shown to inhibit specifically deoxyribonucleic acid (DNA) synthesis in bacteria (14, 40). This compound also inhibits DNA synthesis in mammalian cells and possesses antineoplastic properties. Despite intensive study of the effects of HU on bacterial metabolism over the last few years, considerable disagreement as to the exact mechanism of inhibition of DNA synthesis by this chemical still exists.

It has been suggested by several workers that HU inhibits DNA synthesis by interfering with the formation of deoxyribonucleotide precursors of DNA by the enzyme ribonucleoside

diphosphate reductase (7, 12, 21, 25, 31, 45). Elford (12) has shown that HU drastically inhibits the ribonucleoside diphosphate reductase activity in crude extracts of *Escherichia coli*, but Rosenkranz and Jacobs (41) have presented results contradicting this claim. HU has also been reported to inhibit the activity of purified ribonucleotide reductase from *E. coli* (21). Of the four components of this enzyme system (thioredoxin, thioredoxin reductase, and proteins B1 and B2), only protein B2 is sensitive to this inhibitor. This protein possesses a sharp absorption peak at 410 nm that originates from an iron-containing component of the enzyme (7). Upon incubation with HU, the inactivation of the protein B2 of the *E. coli* ribonucleotide reductase system is accom-

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panied by the loss of the 410-nm absorption peak. These observations led to the suggestion that HU causes an inhibition of DNA synthesis by conversion of the iron-containing component of protein B2 to an inactive form (7, 36). However, addition of iron to purified ribonucleotide reductase does not reverse the inhibitory effects of HU (25). Furthermore, hydroxylamine and hydroxyurethane, neither of which is a specific inhibitor of DNA synthesis, also inhibit purified ribonucleotide reductase activity. In the case of hydroxylamine, it has been shown that the protein B2 is the site of inhibitory action, and the loss of reductase activity is accompanied by a loss of the absorption peak at 410 nm. The effects of HU on DNA synthesis in *E. coli* are completely reversible for at least 2 hr, and this reversal can be accomplished in the absence of protein synthesis (37), whereas the inhibition of purified ribonucleotide reductase is not reversed by removal of the inhibitor (21).

Neuhard (31) and Neuhard and Thomassen (33) found the addition of HU to bacteria to result in a decrease in the size of the acid-soluble deoxyribonucleoside triphosphate pools. Acid-soluble pool studies of Rosenkranz and Jacobs (41), on the other hand, led them to the conclusion that HU does not inhibit the synthesis of deoxyribonucleotides but rather the conversion of deoxyribonucleoside diphosphates to their triphosphate derivatives. To evaluate these conflicting interpretations properly, the effects of HU on the total ribonucleotide and deoxyribonucleotide pools need to be studied. Warner and Hobbs (45) found that in phage T4-infected *E. coli* HU produces an increase in the total ribonucleotide pools and a pronounced decrease in the total deoxyribonucleotide pools.

If ribonucleotide reductase is the only site of action of this inhibitor, then at least a partial reversal might be expected after addition of deoxyribonucleosides to HU-inhibited cells. Such reversals have indeed been observed in some types of mammalian tissue cultures (2, 3, 13, 24, 48), but none has been obtained in bacteria (38). This failure to obtain such a reversal by adding deoxyribonucleosides to bacteria is expected since bacteria, unlike mammalian cells, apparently lack nucleoside kinase activity specific for deoxyadenosine, deoxycytidine, and deoxyguanosine (18).

An alternative hypothesis as to the mechanism of inhibition of DNA synthesis by HU has been proposed by Rosenkranz et al. (38). They suggest that HU inhibits DNA synthesis by causing a disturbance in the structure of the DNA template, thus making it incapable of directing normal DNA replication.

In this paper, we report the results of studies

on the effects of HU on acid-soluble DNA precursor pools and ribonucleotide reductase activity in *E. coli*, performed in an attempt to establish whether HU inhibits the reduction of ribonucleotides in uninfected *E. coli*. In addition, we have investigated the effects of HU on DNA replication and repair in toluene-treated cells and examined the integrity of DNA from HU-treated cells to evaluate the suggestion that HU might be inhibiting DNA synthesis by a mechanism distinct from any effects on ribonucleotide reduction. Also, pulse-chase experiments were done in an attempt to detect any HU-induced metabolic blocks in the pathway of DNA synthesis by intact cells subsequent to the ribonucleoside diphosphate reduction step.

MATERIALS AND METHODS

Media and bacterial strains. H broth (nutrient broth, 8 g [Difco]; peptone, 5 g [Difco]; sodium chloride, 5 g; glucose, 1 g; distilled water, 1 liter), T broth (tryptone, 10 g [Difco]; sodium chloride, 5 g; distilled water, 1 liter), EHA bottom layer agar (agar, 10 g [Difco]; tryptone, 13 g [Difco]; sodium chloride, 8 g; sodium citrate [dihydrate], 2 g; glucose, 1.3 g; distilled water, 1 liter), and EHA top-layer agar (agar, 6.5 g [Difco]; tryptone, 13 g [Difco]; sodium chloride, 8 g; sodium citrate [dihydrate], 2 g; glucose, 3 g; distilled water, 1 liter) (11) were used for growth of overnight bacterial cultures, dilutions, and plating, respectively. M9CA medium (M9 medium of Adams [1] supplemented with 5 μ g of thymidine per ml, 0.2% glycerol, and 0.3% Casamino Acids [Difco] which was added as a charcoal-filtered solution) was used for growth of bacteria throughout this study. *E. coli* B/5, a prototroph, was used in all experiments.

Radioisotopes and scintillation counting. Thymidine-2- 14 C (55.7 mCi/mmol) and uracil-2- 14 C (18.6 mCi/mmol) were obtained from International Chemical and Nuclear Corp., Irvine, Calif. Thymidine-methyl- 3 H (11.3 Ci/mmol), [5- 3 H]cytidine-5-diphosphate (13.5 Ci/mmol) and deoxyadenosine-5'-triphosphate-8- 3 H-tetralithium (5.54 Ci/mmol) were obtained from Schwarz Bioresearch Inc., Orangeburg, N.Y.

2,5-Diphenyloxazole (PPO) and 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) were obtained from Packard Instrument Co., Downer's Grove, Ill.

Two scintillation fluids were used. For nonaqueous materials, a solution containing 4 g of PPO and 0.25 g of dimethyl POPOP per liter of toluene was used. For aqueous solutions, Bray solution (6) was used. The radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer.

Chemicals. HU was purchased from Calbiochem Inc., Wood Dale, Ill. Polyethyleneimine-cellulose-coated plastic sheets for thin-layer chromatography (TLC) were obtained from Brinkman Instruments, Westbury, N.Y. Pancreatic deoxyribonuclease and ribonuclease were purchased from Worthington Biochemical Corp., Freehold, N.J.

Measurement of the effects of HU on cellular metabolism. Cell mass was measured with a Beck-

man DB spectrophotometer at 650 nm. The number of viable cells was determined by plating an appropriate dilution on EHA bottom-layer agar plates. For determination of DNA, ribonucleic acid (RNA), and protein concentrations, cells were withdrawn at various times after addition of HU to log-phase cultures of *E. coli* B/5, and macromolecules were precipitated by adding trichloroacetic acid to a final concentration of 5%. The trichloroacetic acid precipitates were allowed to sit in an ice bath for 30 min, after which they were centrifuged and washed three times with 5-ml quantities of cold 5% trichloroacetic acid solution before being dissolved in 5 ml of 0.1 M NaOH solution. DNA concentration was determined by the indole assay (44); RNA was determined by the orcinol assay (10). Protein content was measured by the method of Lowry et al. (22). Salmon sperm DNA, yeast RNA, and trypsin were used as standards in the above assays.

Ribonucleotide reductase assays. Three liters of exponentially growing cells (approximately 3×10^8 cells/ml in M9CA medium) were chilled by adding crushed ice, harvested by centrifugation, and either used immediately or frozen for up to 1 week for future use. The cell extracts were prepared, enzyme assays performed, and Dowex 50W column chromatography was done as described by Yeh et al. (47). The fractions from the column were analyzed for radioactivity by adding 2 ml of each fraction to 10 ml of Bray solution and counting in a scintillation spectrometer as described earlier.

Acid-soluble pool studies. Acid-soluble pyrimidine pools were labeled by growing log-phase cells of *E. coli* B/5 in the presence of 0.1 μ mole of ^{14}C -uracil (3.2×10^7 counts per min per μ mole; 18.6 mCi/mmole) per ml for 15 min. At the desired intervals, 1-ml samples were withdrawn, and the cells were collected by membrane filtration (Millipore Corp.). The cells were washed on the filters with three 2-ml portions of cold M9CA medium, and the membrane filters (Millipore Corp.) quickly immersed in 5 ml of cold 5% trichloroacetic acid. The filters were vigorously shaken in the trichloroacetic acid solution for 15 seconds and then allowed to stand in an ice bath for 30 min or longer. The trichloroacetic acid precipitates were centrifuged, and the supernatant fluid was extracted 10 times with equal volumes of cold ether before being lyophilized to dryness. The residues were dissolved in 0.1 ml of water, and 10- μ liter samples were placed on PEI-cellulose plates with reference nucleotides (25 nmole of each) for TLC. To estimate the amount of ^{14}C -uracil incorporated into DNA, the trichloroacetic acid precipitates were dissolved in 0.5 ml of 0.3 M KOH and incubated for 18 hr at 30 C. Five ml of 10% trichloroacetic acid was added to each sample, and the precipitates were allowed to sit in an ice bath for 30 min. The precipitates were then centrifuged and washed three times with 5-ml volumes of cold 5% trichloroacetic acid before being dissolved in 2-ml volumes of 0.1 M NaOH. Samples (1 ml) were then dried in scintillation vials and counted.

PEI-cellulose plates were prepared for TLC as described by Randerath and Randerath (34). Before carrying out two-dimensional chromatography to sep-

arate nucleotides, the TLC plates were washed with tris(hydroxymethyl)aminomethane (Tris)-methanol and methanol-water solutions as described by Neuhaud et al. (32) to remove salts and other interfering substances.

Monophosphates were separated by the method of Randerath and Randerath (35). Di- and triphosphates were separated by the method of Neuhaud (30). After chromatographic separation, the plates were dried; the nucleotide-containing (as indicated by ultraviolet absorption) spots were cut out; and the radioactivity in each was counted.

Preparation of labeled DNA. Two identical cultures of *E. coli* B/5 were grown for 6 to 8 generations in M9CA medium supplemented with 0.5 mg of uridine per ml. One culture also contained 0.5 μ Ci of ^{14}C -thymidine per ml, and the second contained 25 μ Ci of ^3H -thymidine per ml. When the cell density reached about 4×10^8 cells/ml, 2-ml samples were withdrawn, filtered on membrane filters (Millipore Corp.), washed with 10 ml of cold M9CA medium, and resuspended in 2 ml of prewarmed M9CA medium. The ^{14}C -labeled cells were added to a medium that also contained 26 mM HU. Both cultures were incubated another 30 min at 30 C with vigorous aeration. The two cultures were then pooled, and the DNA was extracted by using a gentle extraction procedure developed by T. Lash (Ph.D. thesis, Yale University, 1971). The DNA molecules extracted by this procedure have a molecular weight of 1.5×10^6 to 2.5×10^6 .

This extraction procedure involves freezing and thawing cells in the presence of an equal volume of 30% sucrose in 0.1 M Tris-0.1 M ethylenediaminetetraacetic acid (EDTA), pH 8.5, with a small amount (0.1 ml) of 0.5 M NaN_3 . This is followed by lysozyme treatment (final concentration of the enzyme, 4×10^4 units/ml) at 0 C for 15 min. The spheroplasts are lysed by addition of Sarkosyl to a final concentration of 0.1%. RNA is digested with ribonuclease (final concentration, 40 μ g/ml) at 37 C for 30 min. Then Pronase (a freshly prepared solution in 0.1 M Tris-chloride, pH 8.5) is added as an 80 mg/ml solution to achieve a final concentration of 2 mg/ml. Pronase treatment is continued for 15 to 18 hr at 37 C. Before use, both ribonuclease and Pronase were heated to 80 C for 10 min and then quickly chilled in an ice bath to inactivate any contaminating deoxyribonucleases. After the Pronase treatment, the DNA solution is dialyzed against a buffer containing 0.01 M Tris, 0.005 M EDTA, and 0.02 M sodium chloride. (One ml of 95% ethanol is added to each liter of dialysis buffer just before use. The dialysis bags are prepared for use by boiling twice in 0.5% sodium carbonate-0.05% sodium citrate buffer, pH 8.6, and then once in distilled water.) The DNA solution is dialyzed against three consecutive 1-liter volumes of buffer over 24 hr (8 hr in each) at room temperature.

Sucrose density gradient centrifugation. Sucrose was dissolved in 2 M sodium chloride solution (containing 10^{-3} M EDTA) at pH 8.5 (0.01 M Tris) for neutral gradients and at pH 12.2 (0.02 M sodium phosphate) for alkaline gradients. Gradients were always prepared with freshly dissolved solutions of sucrose. Linear sucrose gradients of 2.4 ml each of 5%

(w/v) and 20% (w/v) sucrose solutions were prepared as described by Martin and Ames (23) in 5-ml nitrocellulose tubes that had been pretreated with a silicone preparation (Siliclad) to prevent DNA from sliding to the bottom of the tube (8). All gradients were stored in the cold for at least 60 min before use. The DNA preparations (0.3 ml) were gently layered on the gradients.

Centrifugations were carried out with an SW50.1 Beckman rotor in a Beckman L2-65B centrifuge. All centrifugations were done at 18 C for 15 to 20 hr at 20,000 rev/min. Ten-drop fractions were collected by puncturing a hole in the bottom of the centrifuge tube. The fractions were collected directly on Whatman GF/A glass-fiber discs in scintillation vials and dried at 60 C, and the radioactivity was counted.

DNA synthesis in toluene-treated cells. *E. coli* B/5 cells were grown to a cell density of 5×10^8 to 6×10^8 cells/ml, harvested by centrifugation, and treated with toluene as described by Moses and Richardson (27). DNA-synthesizing capacity was determined as described by Moses and Richardson (28). Assay mixtures containing all the necessary components were prepared in a total volume of 0.18 ml to which was added 0.02 ml of a freshly prepared aqueous solution of HU to obtain the desired final concentration of the inhibitor in each assay tube. The tubes were preincubated at 30 C for 15 min before adding 10^8 to 2×10^8 cells in 0.1 ml of 0.05 M potassium phosphate buffer. DNA synthesis was allowed to proceed for 15 min at 30 C.

Pulse-chase studies. A 16-ml culture was grown in M9CA medium at 30 C with vigorous aeration to a cell density of approximately 4×10^8 cells per ml. ^{14}C -uracil was added to a final concentration of 2 Ci/ml (approximately 0.1 $\mu\text{mole/ml}$), and the cells were incubated for another 25 min. A 1-ml sample was then withdrawn, and the cells were collected on a membrane filter (Millipore Corp.) and washed three times with 2 ml of cold M9CA medium. Thereafter, the filter and the cells were shaken for 15 sec in 0.5 ml of ice-cold 1 M perchloric acid. Immediately after removal of the above sample, two 7-ml samples were withdrawn and washed free from radioactive uracil by membrane filtration (Millipore Corp.). The cells from one of the 7-ml samples were suspended in 7 ml of prewarmed M9CA medium supplemented with 2.5 μmole of cold uracil per ml. The cells from the other sample were suspended in 7 ml of M9CA medium that also contained HU (0.033 M) in addition to cold uracil (2.5 $\mu\text{mole/ml}$). Both cultures were grown at 30 C with aeration. Samples (1 ml) were withdrawn at the desired intervals, and the cells were collected on membrane filters (Millipore Corp.) and washed with cold M9CA. The filters were then quickly immersed in 0.5 ml of 1 M perchloric acid and shaken to precipitate macromolecules. Bovine serum albumin (1 mg) was added to each tube, and the precipitates were allowed to sit in an ice-bath for 60 min. The precipitates were collected by centrifugation, RNA was hydrolyzed, and the radioactivity in DNA was estimated as previously described.

The perchloric acid supernatant fluids were placed in a boiling-water bath for 10 min to hydrolyze

nucleoside polyphosphates to nucleoside monophosphates. The samples were brought to room temperature and neutralized by using 8 M KOH solution containing a small quantity of phenol red as a pH indicator. The precipitates were then centrifuged, and the supernatant fluids were used for the estimation of labeled ribo- and deoxyribonucleotides by TLC. A 0.1-ml sample of this supernatant fluid was withdrawn, and the radioactivity was counted after the addition of 10 ml of Bray solution to estimate the total acid-soluble radioactivity.

PEI cellulose-coated plates were prepared for TLC as described earlier. Ten-microliter amounts of the samples were placed 1.5 cm apart along with 25 nmoles of each reference nucleotide on a PEI-cellulose plate. Subsequent to application of the samples, the TLC plates were washed with Tris-methanol and anhydrous methanol as described by Neuhaed et al. (32). Ribonucleotides were separated from deoxyribonucleotides by one-dimensional chromatography in a solvent composed of 6 g of sodium tetraborate, 3 g of boric acid, 25 g of ethylene glycol, and 70 ml of water. The solvent front was allowed to ascend approximately 15 cm above the origin. In this system, uracil, uridine, cytidine, deoxyuridine, deoxycytidine, and thymidine migrate with a R_f between 0.7 and 0.8. Pyrimidine-containing deoxyribonucleotides (deoxyuridine monophosphate, thymidine monophosphate, and deoxycytosine monophosphate) move with an R_f of about 0.5, and pyrimidine-containing ribonucleotides (cytosine monophosphate and uridine monophosphate) possess a R_f of approximately 0.28. After chromatography, the TLC plates were dried, and the radioactivity in nucleotides was estimated as described earlier.

RESULTS

Effects of HU on bacterial metabolism.

Preliminary studies indicated that the concentration of HU (0.1–0.2 M) commonly used by other workers (31, 37, 41, 42) in studies with *E. coli* resulted not only in an inhibition of DNA synthesis but also in a pronounced inhibition of RNA and protein synthesis. Therefore, the effects of various concentrations of HU on *E. coli* B/5 metabolism, i.e., number of viable cells, cell mass, and DNA, RNA, and protein concentrations, were examined in an attempt to find a concentration that completely inhibits DNA synthesis and increase in number of viable cells but has little or no effect on other metabolic processes.

Increase in the number of viable cells (Fig. 1a) and DNA synthesis (Fig. 1b) are the processes most sensitive to HU. Number of viable cells ceases to show a prolonged increase at any concentration of HU higher than 0.02 M. The effect of HU on number of viable cells is reversible for about 2 hr, after which a slow devitalization begins. The rate of cell death is dependent upon the concentration of HU pres-

ent (Fig. 1a). DNA synthesis is completely (greater than 95%) inhibited by an HU concentration of 0.026 M (Fig. 1b). At none of the HU concentrations used was there a net decrease in the amount of DNA present, indicating that this compound does not induce a significant amount of degradation of DNA to acid-soluble material. A study of the effects of HU on increases in cell mass (Fig. 1c), and RNA (Fig. 1d) and protein (Fig. 1e) concentrations revealed that these processes are much less sensitive to the effects of this inhibitor. Nevertheless, there is a small, but significant, decrease in the rates of increase in cell mass and RNA and protein synthesis with each increase in HU concentration. Therefore, in all further experiments, the lowest HU concentration giving a complete inhibition of DNA synthesis (0.026–0.033 M) was used to minimize the other effects of the inhibitor. Also, treatment was always for less than 2 hr to avoid irreversible damage to the cells.

When the effect of HU on the incorporation of radioactive thymidine into DNA of *E. coli* B/5 was investigated, a significant amount of label was found to be incorporated into trichloroacetic acid-insoluble material in the presence of 0.026 M and even higher concentrations of HU (N. K. Sinha, *unpublished results*). This incorporation probably represents repair synthesis. It may be mentioned however that no difference in the rates of thymidine incorporation was observed in a *polA*⁺ strain and its *polA* mutant derivative.

CDP reductase activities. Since contradicting results have been reported by Elford (12) and Rosenkranz and Jacobs (41) regarding the inhibition of ribonucleotide reductase in crude extracts of *E. coli* by HU, these experiments were repeated in this study (Fig. 2). At HU concentrations of 10⁻³ M and higher, the cytidine diphosphate (CDP) reductase activity present in crude extracts of *E. coli* B/5 was markedly reduced. The degree of inhibition obtained with 0.01 M HU varied from 80 to 100%. Our results are thus in agreement with those of Elford (12) and contradict those of Rosenkranz and Jacobs (41).

Effects of HU on ribonucleotide reduction in vivo. The effect(s) of HU on the relative sizes of the ribo- and deoxy ribonucleotide pools was examined to see whether HU caused an inhibition of ribonucleotide reduction in vivo. The acid-soluble pyrimidine pool was labeled, HU was added, and the amount of radioactivity present in ribo- and deoxyribonucleoside mono-, di-, and triphosphates was examined at various times (Fig. 3a, b, and c; Table 1). After

the addition of HU, the amount of label in ribonucleoside mono- and diphosphates increases, whereas the labeled ribonucleoside triphosphates show an initial increase followed by a decrease in concentration. The deoxyribonucleoside monophosphates are present in quite low amounts, and no change in their levels is seen, but addition of HU results in a decrease in the concentration of deoxyribonucleoside di- and triphosphates. The decrease is especially pronounced in the case of the triphosphates. Table 1 shows the total amounts of labeled uracil- and cytosine-containing nucleotides and their distribution in ribo- and deoxyribonucleotides. It is obvious from these results that both uracil- and cytosine-containing ribonucleotides show an increase in their total amounts and that the deoxyribonucleotides show a decrease in their total concentrations after the addition of HU to bacteria. In addition, the proportion of label present in nucleotides increases in the presence of HU. The decrease in the amount of deoxyribonucleotides would be even more pronounced than those shown in Table 1 if the values expressed were corrected for the observed expansion of the nucleotide pools.

Effects of HU on DNA integrity. A culture of *E. coli* B/5 was divided into two subcultures; one subculture was labeled with ¹⁴C-thymidine and the other with ³H-thymidine. The radioactive precursors were subsequently removed by membrane filtration (Millipore Corp.). The cells with ¹⁴C-labeled DNA were grown in M9CA medium supplemented with HU (final concentration 0.026 M), and those with ³H-labeled DNA were grown in unsupplemented medium, both for an additional 30 min at 30 C. The treated and untreated cells were then pooled, and their DNA was extracted by a gentle extraction procedure. Mixing the cells before extraction insured that any differences in the DNA of the two subcultures would be due to the effect(s) of HU treatment rather than to any differences in the handling procedure. Induction of any breaks in the DNA of the treated cells due to HU treatment will result in this DNA having a lower, average molecular weight than the DNA from untreated cells. The cells with ¹⁴C-labeled DNA, rather than the ³H-labeled ones, were treated with HU because tritium decay is known to cause single- and double-strand breaks in DNA (39). The labeled DNA mixture was analyzed for HU-induced breaks in the structure by sedimentation through neutral and alkaline gradients. The results of sucrose density gradient sedimentation are shown in Fig. 4a and b. The ³H-labeled (control) DNA is re-

solved into two components in neutral sucrose gradients (Fig. 4a). The faster of these cosediments with the band containing the HU-treated DNA. The slower sedimenting ^3H -labeled component consists of DNA of a lower molecular weight presumably formed as a result of the induction of double-strand breaks by ^3H decay. The absence of any shoulders or lighter peaks in the curve representing the distribution of ^{14}C -labeled DNA suggests that HU did not cause any double-strand breaks.

In alkaline gradients (Fig. 4b), the ^{14}C -labeled DNA sediments as a single band that cosediments with only a small fraction of the ^3H -labeled DNA. In neutral sucrose gradients, approximately half the ^3H -labeled DNA cosediments with the ^{14}C -labeled DNA. In alkaline sucrose-gradients, on the other hand, the bulk of ^3H -labeled DNA has a slower sedimentation rate than the ^{14}C -labeled DNA. The ^3H -labeled

DNA appears to possess considerably more single-strand breaks than double-strand breaks. These results are in agreement with the observations of Rosenthal and Fox (39) who also found that tritium decay results primarily in the induction of single-stranded nicks, and only a small fraction of the decays result in double-stranded breaks.

Because of the ambiguity introduced by the induction of a large number of breaks in the tritium-labeled DNA, control experiments were performed which were identical to those described above except that neither the ^3H - nor the ^{14}C -labeled cultures were exposed to HU (Fig. 4c and d). The distributions of ^3H - and ^{14}C -labeled DNA in both neutral and sucrose gradients are essentially the same whether the ^{14}C -labeled cells are exposed to HU or not. These results strongly suggest that exposure of cells to HU for up to 0.5 hr has little or no effect

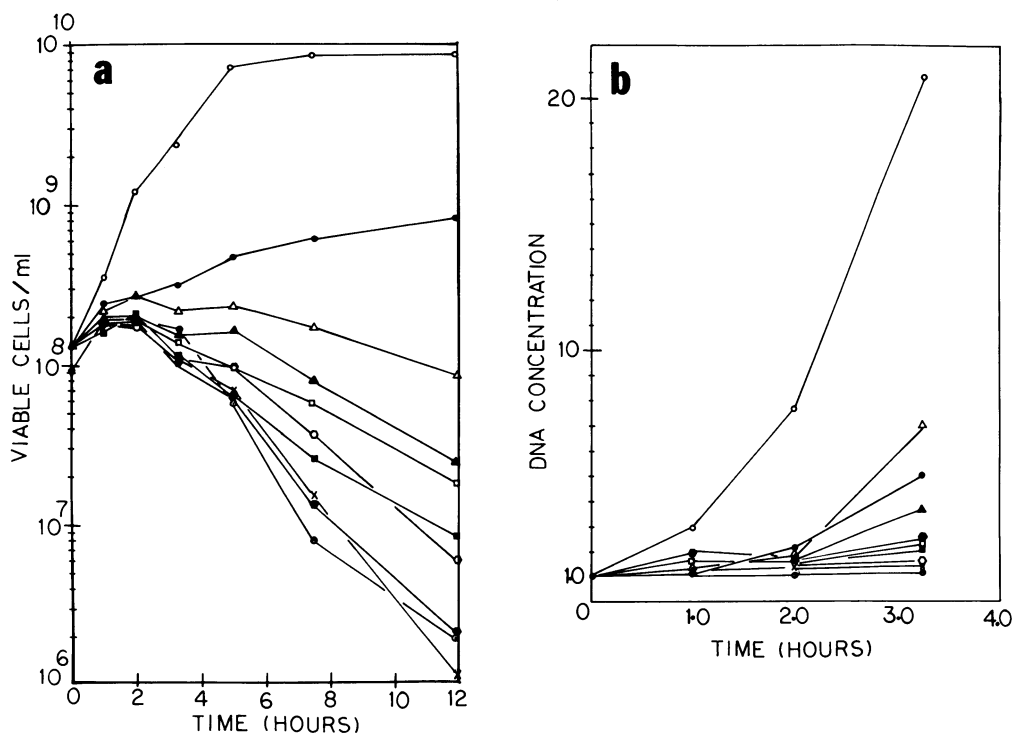


FIG. 1a, b

FIG. 1. Effects of various concentrations of hydroxyurea on increases in (a) number of viable cells, (b) DNA concentration, (c) cell mass, (d) RNA concentration, and (e) protein concentration of *E. coli* B/5 at various times after addition of the inhibitor. The concentrations of HU used were \circ , no HU; \bullet , $6.6 \times 10^{-3} \text{ M}$; Δ , $1.32 \times 10^{-2} \text{ M}$; \blacktriangle , $2 \times 10^{-2} \text{ M}$; \square , $2.6 \times 10^{-2} \text{ M}$; \blacksquare , $3.3 \times 10^{-2} \text{ M}$; \bullet , $4 \times 10^{-2} \text{ M}$; \circ , $5.3 \times 10^{-2} \text{ M}$; \times , $6.6 \times 10^{-2} \text{ M}$; \circ , $1.32 \times 10^{-1} \text{ M}$. The DNA, RNA, and protein concentrations were determined using indole, orcinol, and Folin assays, respectively. All concentrations of DNA, RNA, and protein are expressed relative to the concentration existing at the time of addition of the inhibitor.

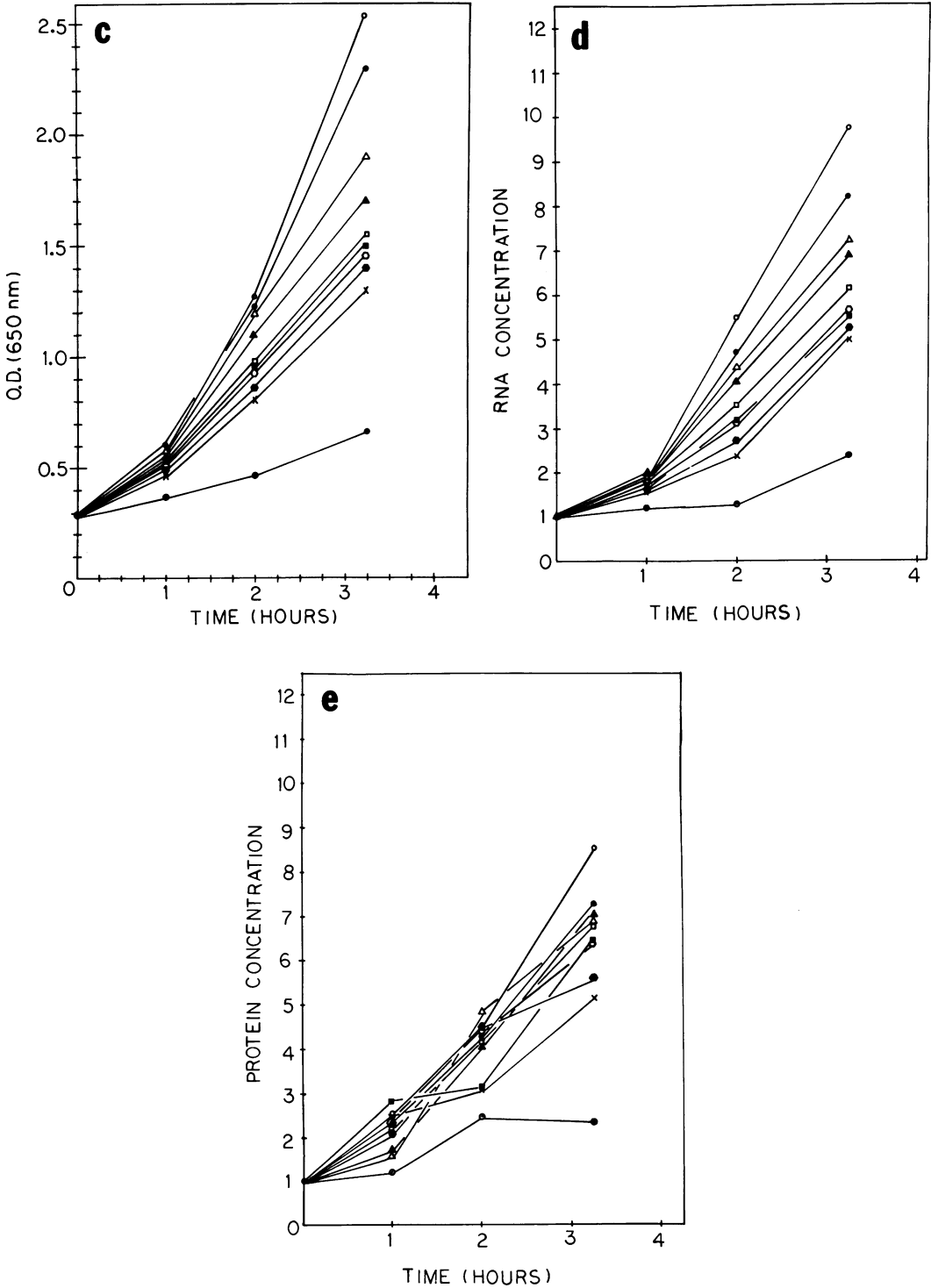


FIG. 1c-e

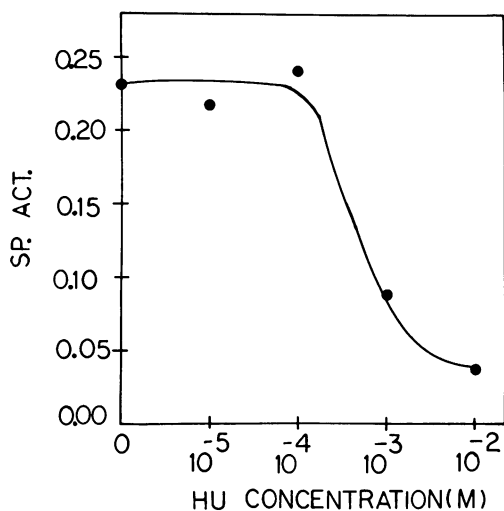


FIG. 2. Ribonucleotide reductase activity in crude extracts of *E. coli* B/5 in the presence of various concentrations of HU. One unit of enzyme is defined as the formation of 1 nmole of deoxycytidine diphosphate (dCDP) from CDP in 20 min at 30 C. Specific activity is the number of units of enzyme per milligram of protein. The radioactive CDP used as substrate in these assays contained approximately 0.1% radioactive dCDP as impurity. All activity calculations were corrected for this impurity.

on the integrity of DNA.

DNA synthesis in toluene-treated cells. The following experiments were done to see whether the inhibition of DNA synthesis during growth of cells in the presence of HU is due to any effects of the inhibitor at the DNA polymerization step.

It has been shown (28) that it is possible to make *E. coli* cells permeable to deoxyribonucleoside triphosphates by treating them with toluene. It has also been demonstrated (26, 28) that DNA synthesis, obtained in cells treated in this manner after addition of the four deoxyribonucleoside triphosphates and adenosine triphosphate (ATP), exhibits many of the properties expected of true DNA replication rather than repair synthesis. Use of this technique has been made to examine the hypothesis that HU inhibits DNA synthesis by modifying DNA such that it can no longer serve as a template for replicative synthesis. It was found that, at concentrations of HU as high as 0.132 M, no effect could be detected on replicative or repair synthesis of DNA in toluene-treated cells (Fig. 5).

Pulse-chase experiment. The fate of deoxyribonucleotides synthesized prior to the addition of HU to *E. coli* B/5 cells was studied in an attempt to discover any metabolic blocks in-

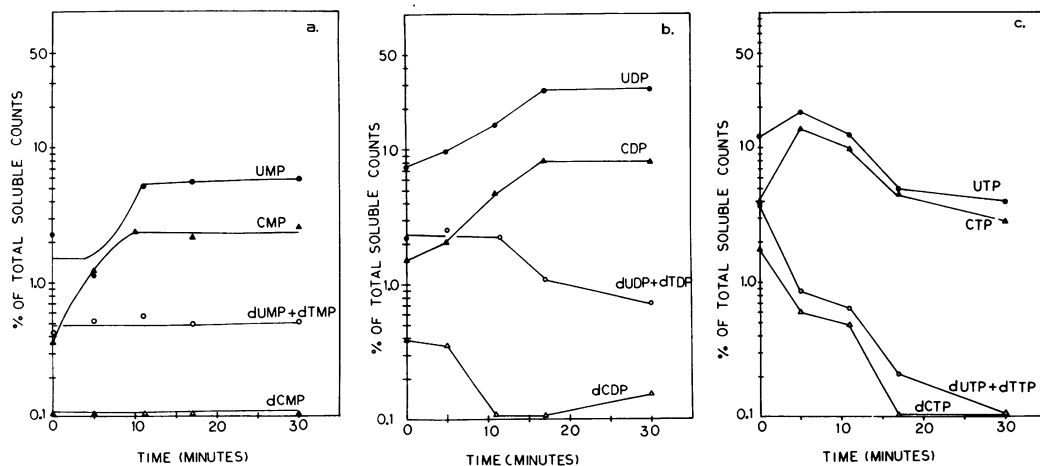


FIG. 3. Effect of 0.033 M HU on the label in acid-soluble pyrimidine pools of *E. coli* B/5. Acid-soluble pools were labeled by growing log-phase cells with 2 μ Ci (approximately 0.1 μ mole) of 14 C-uracil per ml for 15 min and adding HU at the end of this period. The time is expressed as minutes after addition of HU. The nucleotides were separated by thin-layer chromatography on PEI-cellulose-coated plates. The chromatographic procedure used does not separate deoxyuridine- and thymidine-containing nucleotides. Their concentrations are, therefore, expressed as the sum of the concentration of the two. The total acid-soluble counts were typically between 4×10^4 and 5×10^4 counts/min. Any nucleotide with a concentration lower than 0.1% of the total soluble counts/min could not be detected, and all values lower than 0.1% have been expressed as 0.1%. a, Monophosphates; b, diphosphates; c, triphosphates.

TABLE 1. *Effects of addition of hydroxyurea on the total labeled pyrimidine ribo- and deoxyribonucleotide concentrations in E. coli B/5 cells^a*

Min after addition of HU ^b	Radioactivity ^c					
	Uracil-containing nucleotides			Cytosine-containing nucleotides		
	Total ribo-nucleotides ^d	Total deoxyribo-nucleotides ^e	Total nucleotides	Total ribo-nucleotides ^f	Total deoxyribo-nucleotides ^g	Total nucleotides
0.0	21.63	6.42	28.05	6.12	2.22	8.34
5.0	29.11	3.99	33.10	17.11	0.95	18.06
11.0	33.19	3.43	36.62	16.91	0.58	17.49
17.0	37.73	1.62	39.35	14.93	0.10	15.03
30.0	37.70	1.24	38.94	13.19	0.16	13.35

^a Cells prelabeled by growth for 15 min with ¹⁴C-uracil.

^b Hydroxyurea was added at 0 time to a final concentration of 0.033 M.

^c All the values are expressed as percentage of total acid-soluble counts. Values are based on the data shown in Fig. 4.

^d Uridine mono-, di-, and triphosphates.

^e Deoxyuridine mono-, di-, and triphosphates.

^f Cytidine mono-, di-, and triphosphates.

^g Deoxycytidine mono-, di-, and triphosphates.

duced in the DNA synthetic pathway subsequent to the synthesis of deoxyribonucleotides. After labeling of the pyrimidine pools with ¹⁴C-uracil, the radioactive precursor was removed, and an excess of cold uracil was added to dilute any residual radioactive uracil, thus preventing the synthesis of nascent radioactive nucleotides. The concentration of labeled ribo- and deoxyribonucleotides and the amount of radioactive DNA made at various times after the cold uracil chase were compared in the presence and absence of HU. If HU inhibited any metabolic processes leading to DNA synthesis subsequent to the synthesis of deoxyribonucleotides, then the amount of radioactive deoxyribonucleotides present would be expected to remain constant after the cold uracil chase in the presence of HU. In the absence of HU, on the other hand, the amount of radioactivity present in deoxyribonucleotides would be expected to decrease rapidly as the radioactive nucleotides are utilized for DNA synthesis. Furthermore, this chase of radioactive deoxyribonucleotides into DNA would result in the synthesis of a larger quantity of radioactive DNA in the absence of HU than in its presence. However, if HU did not inhibit any steps subsequent to the reduction of ribonucleotides then both the rate of decrease of radioactivity in deoxyribonucleotides and the rate of

increase of radioactivity in DNA would be unaffected by the presence of HU in these chase experiments.

The rate of decrease of radioactivity in deoxyribonucleotides during the cold uracil chase is essentially the same whether HU is present or not (Fig. 6). Moreover, the rate at which the radioactivity in DNA increases during the chase is also unaffected by the presence of HU (Fig. 6b). These results suggest that none of the processes necessary for DNA synthesis subsequent to the reduction of ribonucleotides are affected by HU.

DISCUSSION

The results presented in this paper show that HU inhibits the activity of the enzyme ribonucleoside diphosphate reductase in vitro. The pyrimidine pool labeling experiments indicate that this is also true in vivo. It is well known that the same enzyme complex catalyzes the conversion of all four ribonucleoside diphosphates into the corresponding deoxy components (for a review of this evidence, see reference 36). Any inhibition of this enzyme will, therefore, affect the formation of all four deoxyribonucleotides.

Our results contradict the results of Rosenkranz and Jacobs (41) on two points. First,

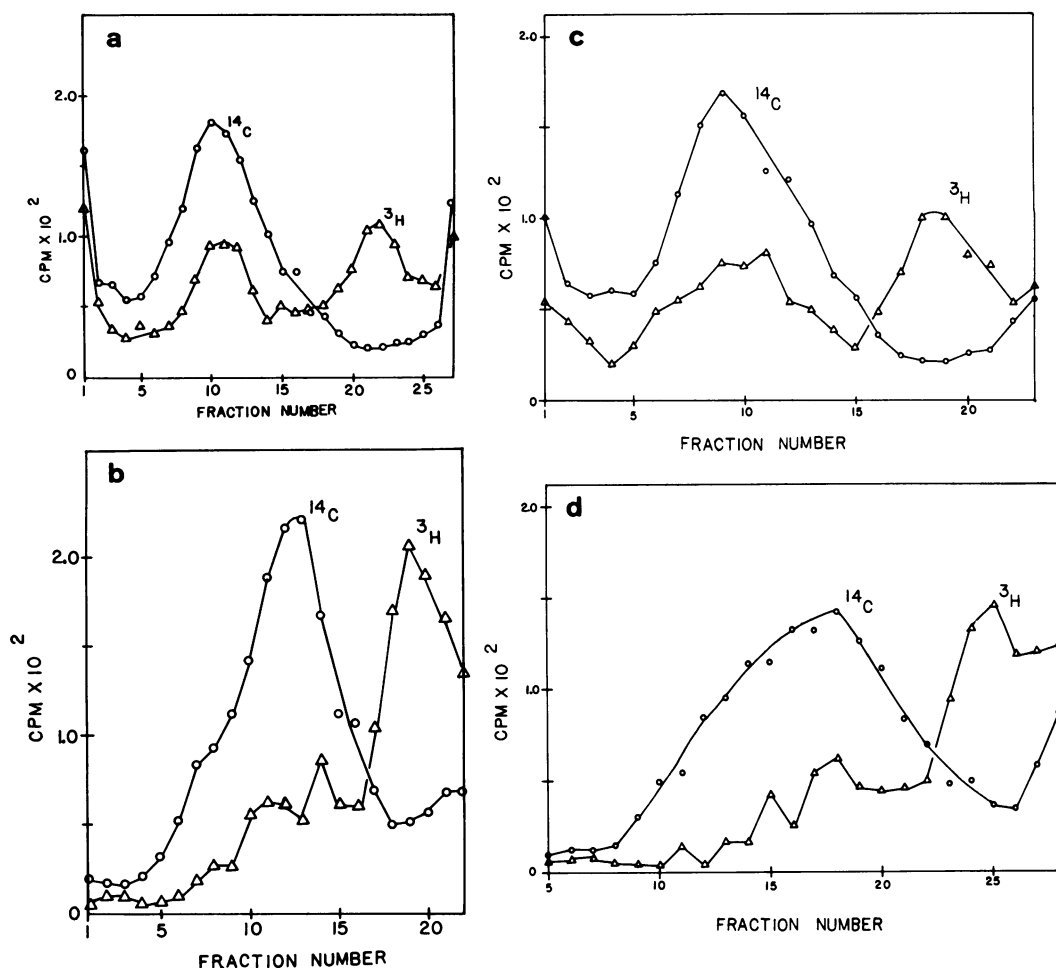


FIG. 4. Sucrose density gradient sedimentation patterns of mixtures of ^3H -labeled DNA from untreated cells and ^{14}C -labeled DNA from HU-treated (a and b) (0.026 M for 30 min) and untreated (c and d) cells. a and b, The DNA of the HU-treated cells was prelabeled by growth for many (6-8) generations in the presence of thymidine-2- ^{14}C (0.5 $\mu\text{Ci/ml}$); the DNA of the untreated cells was prelabeled by growing in the presence of thymidine-methyl- ^3H (25 $\mu\text{Ci/ml}$). c and d, Same as (a and b), but neither culture exposed to HU. Ten-drop fractions were collected after a hole was made in the bottom of the centrifuge tube. a and c, Neutral sucrose gradients; b and d, alkaline sucrose gradients. Symbols: O, ^{14}C ; Δ , ^3H .

Rosenkranz and Jacobs found that HU does not affect the *in vitro* reduction of CDP to deoxy-CDP by crude extracts of *E. coli*. The concentration of HU used by them (0.20 M) is substantially higher than was found necessary to completely inhibit CDP reductase activity both in our experiments and in those of Elford (12). To demonstrate ribonucleotide reductase activity in *E. coli* extracts, it is necessary to use cell extracts with very high protein concentration (in our experiments, the protein concentration in cell extracts was always 20 mg/ml or higher) and a fairly large amount of radioactivity in the substrate (e.g., 5×10^5 counts per min of

^3H -CDP in each assay). It is not clear why Rosenkranz et al. failed to detect any inhibition of ribonucleotide reductase by HU; however, it might be pointed out that they used relatively low amounts of radioactive substrate (^{14}C -CDP; 6×10^3 counts/min).

On the basis of their pool size studies, Rosenkranz and Jacobs (41) concluded that the metabolic block caused by HU is in the conversion of deoxyribonucleoside diphosphates to deoxyribonucleoside triphosphates. The results of our pool labeling studies show that not only does the deoxyribonucleoside triphosphate concentration decrease after HU addition but the

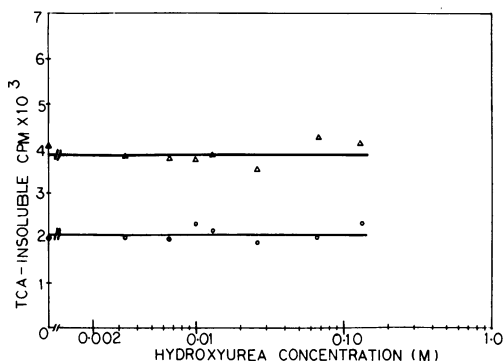


Fig. 5. Replicative and repair synthesis of DNA in toluene-treated cells of *E. coli* B/5. Replicative synthesis (Δ) was measured in the presence of ATP (final concentration 1.3 mM). For measurement of repair synthesis (\circ), ATP was omitted, and pancreatic deoxyribonuclease was added to a final concentration of 0.3 μ g/ml. The incorporation of 8.6×10^3 counts/min into DNA represents an incorporation of 1.0 nmole of ATP.

deoxyribonucleoside diphosphate concentration also, instead of increasing, shows a decline. Moreover, a block in the conversion of deoxyribonucleoside diphosphates to deoxyribonucleoside triphosphates, as proposed by Rosenkranz and Jacobs (41), should leave the ribonucleotide reductase reaction unaffected and result in an increase in the concentration of deoxyribonucleotides relative to ribonucleotides, especially since the concentration of HU used in our study allows a fairly high level of RNA synthesis. At the observed high rate of RNA synthesis, the rate of utilization of newly synthesized ribonucleotides should closely approximate the rate of utilization prior to the addition of HU. The observation that the addition of HU results in net increase in the ribonucleotide concentration along with a marked decrease in the total deoxyribonucleotide concentration strongly suggests that HU induces a block at the ribonucleotide reductase level. These results are not consistent with a dXDP to a dXTP block (where X represents the unknown base). It should be pointed out that the conversion of dXDP to dXTP in *E. coli* is catalyzed by a nonspecific enzyme(s) that also catalyzes the conversion of ribonucleoside diphosphates to ribonucleoside triphosphates (20). Therefore, if HU induced a metabolic block of the kind proposed by Rosenkranz and Jacobs (41), it would be expected to affect both RNA and DNA synthesis equally. Our results indicate that DNA synthesis is much more sensitive to HU than is RNA synthesis.

In spite of the fact that our results clearly

show that the reduction of ribonucleoside diphosphates is inhibited by HU, if there are other metabolic pathways leading to the synthesis of deoxyribonucleotides, then the inhibition of ribonucleoside diphosphate reductase alone might not be sufficient to explain the observed complete inhibition of DNA synthesis by HU. However, there has been an extensive amount of work on this aspect of nucleotide metabolism in *E. coli*, and no other enzyme system capable of synthesizing deoxyribonucleotides has yet been discovered. The results of in vivo labeling experiments indicate that all of the deoxyribonucleotides are made by reduction of ribonucleotides without cleavage of the base-sugar bonds, apparently by a reaction catalyzed by the enzyme ribonucleoside diphosphate reductase (19).

If it is assumed that ribonucleotide reductase is really necessary for DNA synthesis, then the inhibition of this enzyme by HU is sufficient to explain its inhibitory effect on DNA synthesis. However, it would still be very possible that HU might also be affecting other steps involved in DNA synthesis or, in fact, the DNA template itself.

We have failed to find evidence for the suggestion that HU inhibits DNA synthesis by causing a breakdown of the DNA template. No single- or double-stranded breaks were detected in DNA from cells that had been treated with HU concentrations sufficient to completely inhibit DNA synthesis. Considerable evidence indicates that, upon prolonged incubation of *E. coli* cells with HU, it does induce double-strand breaks in DNA (17, 42). There is some evidence which suggests that during such prolonged incubation HU is slowly converted to related

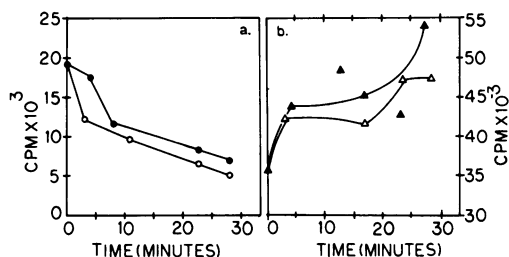


Fig. 6. Pulse-chase experiments. The effect of hydroxyurea on the ability of *E. coli* B/5 cells to incorporate 14 C-labeled deoxyribonucleotides into DNA after the addition of a 25-fold excess of cold uracil. All cells were prelabeled by growth for 25 min in 14 C-uracil (2 μ Ci/ml). Time is expressed as minutes after the addition of cold uracil chase. a, Deoxyribonucleotides; b, DNA. Symbols: \bullet and Δ , cold uracil chase alone; \circ and Δ , cold uracil chase in the presence of 0.033 M HU.

compounds such as *N*-hydroxyurethan and carbamoyloxyurea, and that these are the real causative agents of double-strand breaks in DNA. Purified carbamoyloxyurea causes extensive breakdown of DNA (43). *N*-hydroxyurethan, which is lethal to *E. coli*, also causes extensive degradation of the DNA template and devitalization of *E. coli* cells (29). Both of these effects are evident during prolonged exposure of cells to HU. It seems possible that devitalization of cells and DNA breakdown in cells exposed to HU for prolonged periods of time is not actually due to HU but rather to its derivatives such as carbamoyloxyurea and hydroxyurethan. The inhibition of DNA synthesis by HU, in contrast to the induction of breaks in DNA, is evident within a few minutes after the addition of fairly low concentrations of inhibitor to cells.

DNA synthesis in toluene-treated cells has been shown to result in a semiconservatively replicated product made at a rate of synthesis close to the *in vivo* rate of replication (28). More importantly, DNA synthesis in toluene-treated cells of *E. coli* mutants with temperature-sensitive blocks in DNA replication is also temperature sensitive (26). In addition, DNA synthesis occurs normally in toluene-treated cells of a *polA* mutant of *E. coli* which lacks the predominant polymerase present in wild-type *E. coli* cells (28). The above observations support the proposal that the DNA synthesis occurring in toluene-treated cells in the presence of ATP is really true DNA replication. The observation that HU concentrations as high as 0.13 M have no detectable effect on ATP-dependent DNA synthesis in toluene-treated cells indicates that HU has no effect(s) on DNA synthesis at the DNA polymerization level, either by altering the DNA template or by interfering with any of the other factors needed for true replicative polymerization.

The results of the soluble-pool size studies show that HU causes a drastic reduction in the concentrations of deoxyribonucleotides synthesized. Moreover, the pulse-chase studies show that deoxyribonucleotides synthesized prior to the addition of HU can be utilized for DNA synthesis at the normal rate in the presence of HU. These results lead us to the conclusion that all steps essential for DNA synthesis subsequent to the reduction of ribonucleotides are unaffected by HU. The observations that DNA synthesis can occur normally in toluene-treated cells in the presence of fairly high concentrations of HU and that *in vivo* all steps necessary for DNA synthesis beyond the reduction of ribonucleotides proceed normally in the pre-

sence of HU, along with the evidence discussed below, suggest that HU inhibits DNA synthesis in *E. coli* solely by inhibiting ribonucleotide reductase.

In T4-infected *E. coli*, even though *de novo* synthesis of deoxyribonucleotides is completely inhibited by HU, the deoxyribonucleotides generated by the breakdown of the host chromosome are quite efficiently reutilized to synthesize progeny phage DNA in the presence of high concentrations of HU (45). The average yield is 14 phage particles per infected cell in the presence of HU. This synthesis of phage DNA and viral particles cannot be attributed to a limited amount of escape synthesis or to the presence of some other less efficient mechanism of deoxyribonucleotide synthesis because no phage DNA or viral particles are made in the presence of HU if bacteriophage T4 mutants unable to carry out breakdown of the host genome are used (46).

Lactobacillus leichmanii possesses a ribonucleotide reductase system quite different from the one found in *E. coli*. The reduction of ribonucleotides takes place at the triphosphate level and it is mediated not by an iron-containing component but by a cobalt-containing coenzyme (4, 5, 15). A similar enzyme system presumably exists in *Rhizobium* (9). The *Lactobacillus* ribonucleotide reductase is not sensitive to HU (12), and these organisms are resistant to HU.

A recent study of the metal-binding properties and antitumor activity of several 1-substituted-3-hydroxyureas by Harmon et al. (16) revealed that only those compounds capable of forming complexes with Fe^{3+} possessed antitumor activity. The correlation between the ability to bind iron and antimicrobial and antitumor properties along with the findings of Brown et al. (7), indicating that HU inhibits purified ribonucleotide reductase of *E. coli* by interacting with the iron-containing component of the protein B2, also provide indirect evidence suggesting that the primary, if not the only, effect of low concentrations of HU on DNA synthesis in *E. coli* (at least for approximately the first 2 hr of exposure) is the result of its inhibition of ribonucleoside-diphosphate reductase.

The observation that the addition of deoxyribonucleosides does not reverse the inhibition caused by HU (38) does not exclude the possibility that ribonucleotide reductase is the major, if not the only, site of inhibition of DNA synthesis in *E. coli*. The externally added deoxyribonucleosides have to be phosphorylated before they can be used in DNA synthesis. Even though *E. coli* cells do possess

thymidine kinase activity, it has been found that they lack deoxyadenosine, deoxycytidine, and deoxyguanosine kinase activities (18). Some mammalian systems may possess these kinases, thus explaining the reversal of the effects of HU by the addition of deoxyribonucleosides observed in mammalian tissue cultures (2, 3, 14, 24, 48). The fact that addition of iron to HU-treated purified ribonucleotide reductase in vitro does not restore the activity of the enzyme (25) may simply mean that the proper conditions for the activation of the enzyme in vitro have not been achieved.

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